INFECTION AND IMMUNITY, Apr. 2010, p. 1650–1658 Vol. 78, No. 4
0019-9567/10/$12.00 doi:10.1128/IAI.00001-10

Contribution of *Candida albicans* Cell Wall Components to Recognition by and Escape from Murine Macrophages[▽]

C. G. J. McKenzie, U. Koser, L. E. Lewis, J. M. Bain, H. M. Mora-Montes, R. N. Barker, N. A. R. Gow,† and L. P. Erwig†*

College of Life Sciences & Medicine, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom

Received 2 January 2010/Returned for modification 16 January 2010/Accepted 26 January 2010

The pathogenicity of the opportunistic human fungal pathogen *Candida albicans* depends on its ability to escape destruction by the host immune system. Using mutant strains that are defective in cell surface glycosylation, cell wall protein synthesis, and yeast-hypha morphogenesis, we have investigated three important aspects of *C. albicans* innate immune interactions: phagocytosis by primary macrophages and macrophage cell lines, hyphal formation within macrophage phagosomes, and the ability to escape from and kill macrophages. We show that cell wall glycosylation is critically important for the recognition and ingestion of *C. albicans* by macrophages. Phagocytosis was significantly reduced for mutants deficient in phosphomannan biosynthesis (*mmn4*Δ, *pmr1*Δ, and *mnt3 mnt5*Δ), whereas O- and N-linked mannan defects (*mnt1*Δ *mnt2*Δ and *mns1*Δ) were associated with increased ingestion, compared to the parent wild-type strains and genetically complemented controls. In contrast, macrophage uptake of mutants deficient in cell wall proteins such as adhesins (*ece1*Δ, *hwp1*Δ, and *als3*Δ) and yeast-locked mutants (*clb2*Δ, *hgc1*Δ, *cph1*Δ, *efg1*Δ, and *efg1*Δ *cph1*Δ), was similar to that observed for wild-type *C. albicans*. Killing of macrophages was abrogated in hypha-deficient strains, significantly reduced in all glycosylation mutants, and comparable to wild type in cell wall protein mutants. The diminished ability of glycosylation mutants to kill macrophages was not a consequence of impaired hyphal formation within macrophage phagosomes. Therefore, cell wall composition and the ability to undergo yeast-hypha morphogenesis are critical determinants of the macrophage's ability to ingest and process *C. albicans*.

Invasive *Candida albicans* infection can represent a serious clinical complication, especially in patients with an impaired immune system. Candidemia has an incidence of between 1.1 and 24 cases per 100,000 individuals and an associated mortality rate of more than 30% (42). It seems unlikely that antifungal agents will have an impact on the grim mortality statistics for systemic fungal infections without the aid of new clinical approaches, such as combining chemotherapy with immunotherapy. However, augmenting the ability of the immune system to eliminate a pathogen requires sophisticated understanding of the molecular mechanisms involved in pathogen recognition and the ensuing immune response.

The first point of contact with cells of the innate immune system is the fungal cell wall, and therefore the fungal cell wall plays an important role in directing the immune response to fungal infection (27). It is a dynamic, highly organized organelle that determines both the shape of the fungus and its viability. The core structure of the *C. albicans* fungal cell wall is composed of a skeleton of polysaccharide fibrils composed of β-(1,3)-glucan that is covalently linked to β-(1,6)-glucan and chitin [a β-(1,4)-linked polymer of *N*-acetylglucosamine] and is designed to function as a robust exoskeleton and a scaffold for the external protein layer. This outer layer consists of highly glycosylated mannoproteins that are modified by N-linked (5) and O-linked mannosylation and phosphomannosylation (8, 27).

Host defense against systemic candidiasis relies mainly on the ingestion and elimination of fungal cells by cells of the innate immune system, especially neutrophils and macrophages (12, 27, 38). Macrophages have direct antimicrobial activity against such organisms and also promote antigen presentation, polysaccharide sequestration, and production of cytokines and chemokines (38). Conversely, there is also evidence that persistent infection is associated with the intracellular residence of yeast cells in macrophages. Furthermore, infected circulating macrophages can transfer pathogenic fungi and cause dissemination of infection. Fungi engage different pattern recognition receptors (PRR) to activate specific arms of innate host defense (29). For example, recognition of *C. albicans* by monocytes and macrophages has been shown to be mediated by at least four systems that sense fungal pathogen-associated molecular patterns (PAMPs) of the *C. albicans* cell wall: recognition of N-linked mannans by the mannose receptor (MR), O-linked mannans by Toll-like receptor 4 (TLR4), β-glucans by dectin-1/TLR2, and β-mannosides by galectin-3/TLR2 complexes (28). More recently, additional PRRs have been shown to contribute to *C. albicans* recognition, including the scavenger receptors CD36 and SCARF1 (22), TRL9 recognition of nucleic acids (23), and the C-type lectin mincle (40). The multiplicity of these fungal cell wall-macrophage receptor interactions raises questions as to their relative importance and whether they have differential roles in different cell types and at different stages of infection.

The use of antibodies or soluble mediators to block macro-

* Corresponding author. Mailing address: Division of Applied Medicine, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom. Phone: 44 1224 550526. Fax: 44 1224 555766. E-mail: L.P.Erwig@abdn.ac.uk.

† N. A. R. Gow and L. P. Erwig contributed equally to the paper.

▽ Published ahead of print on 1 February 2010.

TABLE 1. *C. albicans* mutant strains used in this study

Description of strain	Strain	Defect	Reference
CAI4+CIP10	NGY152		26
Glycosylation mutants of *C. albicans*			
*mnt1*Δ *mnt2*Δ	NGY111	O mannosylation	25
*mnt1*Δ *mnt2*Δ::*MNT1*	NGY335		25
*mnt3*Δ *mnt5*Δ	NGY1227	Phosphomannosyltransferase *N*-mannan modification	15
*mnt3*Δ *mnt5*Δ::*MNT3*	NGY1228		15
*mnt3*Δ *mnt5*Δ::*MNT5*	NGY1229		15
*mns1*Δ	HMY5	α1,2-Mannosidase, terminal stage N mannosylation	24
*mns1*Δ::*MNS1*	HMY6		24
*mnn4*Δ	CDH15	Phosphomannan synthesis	16
*mnn4*Δ::*MNN4*	CDH13		16
*pmr1*Δ	NGY355	Phosphomannan synthesis N and O mannosylation	1
*pmr1*Δ::*PMR1*	NGY356		1
C. albicans hyphal cell wall protein mutants			
*ece1*Δ	CAF6-8	Hyphal cell wall protein	4
*als3*Δ	CAYF178U	Surface adhesin	30
*als3*Δ::*ALS3*	CAYF178U		30
*hwp1*Δ	CAH7 1A1E2	Hyphal cell wall adhesin	30
*hwp1*Δ::*HWP1*	CAHR3		30
C. albicans hypha-deficient mutants			
*clb2*Δ	YJB8447	B-type mitotic cyclin	3
*hgc1*Δ	WYZ12.2	G_1 cyclin	44
*efg1*Δ	CA79	cAMP pathway	19
*cph1*Δ	JKC19	MAP kinase cascade	19
*efg1*Δ *cph1*Δ	HLC54	cAMP and MAP kinase	19

phage receptors and the study of receptor knockout macrophages have been informative (7, 14, 18, 28, 35), but these approaches have the inherent limitation of manipulating the host cell while attempting to study the host immune response. Furthermore, since the fungal cell wall is potentially able to trigger many different PRR interactions simultaneously, it is difficult to address the overall contribution of specific *C. albicans* cell wall components to the outcome of the host's immune response (27). Fortunately, the genetic analysis of the biosynthesis of the *C. albicans* cell wall has led to the development of an extensive collection of genetically and phenotypically characterized isogenic mutants of *C. albicans* depleted in specific cell wall components (1, 2, 16, 20, 25, 31). Uptake of *C. albicans* mutants defective in O- and N-linked mannosylation by human mononuclear cells or murine macrophages has previously been shown to diminish cytokine production (14, 28). These *in vitro* findings are relevant to *C. albicans* infections *in vivo*, since *C. albicans* mutants with defects in cell wall mannosyl residues are also less virulent in experimental disseminated candidiasis (1, 2, 24, 25). The vast majority of such mutants have not been formally assessed in terms of recognition by and escape from macrophages.

Here we have conducted a comprehensive analysis of the contribution of *C. albicans* cell wall glycosylation, cell wall proteins, and the ability to switch from yeast to hyphal forms to the fungus-macrophage interaction. For this purpose, we have taken advantage of a large collection of genetically and phenotypically characterized isogenic mutants of *C. albicans* depleted in specific cell wall components or impaired in morphogenic switching. We have assessed three important determinants of *C. albicans* pathogenicity, which hitherto have not been thoroughly studied, namely, phagocytosis by primary murine macrophages and macrophage cell lines, hyphal formation within macrophage phagosomes, and the ability to escape from and kill macrophages. Our data show that specific fungal morphological properties are associated with defined effects on the macrophage's ability to ingest and process *C. albicans*.

MATERIALS AND METHODS

Macrophage cell culture. J774.1 murine macrophages (European Collection of Cell Culture) were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Group, Ltd., Braine-l'Alleud, Belgium), supplemented with 10% (vol/vol) fetal calf serum (FCS; Biosera, Ringmer, United Kingdom), 2% (wt/vol) penicillin and streptomycin antibiotics (Invitrogen, Ltd., Paisley, United Kingdom) and 1% L-glutamine (Invitrogen), in tissue culture flasks (Nagle Nunc, International, Hereford, United Kingdom) at 37°C and 5% (vol/vol) CO_2.

Bone marrow-derived macrophages (BMDM) were obtained by our standard method as previously described (9, 21). BALB/c mouse femurs were flushed aseptically with complete medium to obtain bone marrow cells, through a 25-gauge needle. The single-cell suspension was cultured for 5 days at 37°C at 5% CO_2 in 75-ml tissue culture flasks (Corning Glass, Corning, NY) in DMEM containing 100 U/ml penicillin or 100 U/ml streptomycin, 2 mM glutamine, 10% (vol/vol) heat-inactivated FCS, and 10% L929-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF).

***Candida albicans* yeast cell culture.** *C. albicans* strains were obtained from glycerol stocks and plated on SC-UHTLR plates consisting of yeast nitrogen base-amino acids (Formedium, Norfolk, United Kingdom), technical agar (Oxoid, Cambridge, United Kingdom), 1 M NaOH solution (BDH Chemicals, VWR International, Leicestershire, United Kingdom), double-distilled H_2O, 1% (wt/vol) adenine hemisulfate solution (Sigma, Dorset, United Kingdom), 40% (wt/vol) glucose solution (Fisher Scientific, Leicestershire, United Kingdom), and 4% SC −Ura dropout solution (Formedium). A single colony of *C. albicans* yeast cells was added from SC-UHTLR plates to 5 ml of SC-UHTLR plus 5% glucose medium (Fisher Scientific) and incubated overnight at 30°C in a shaking incubator at 200 rpm. In selected experiments, *C. albicans* yeast cells were

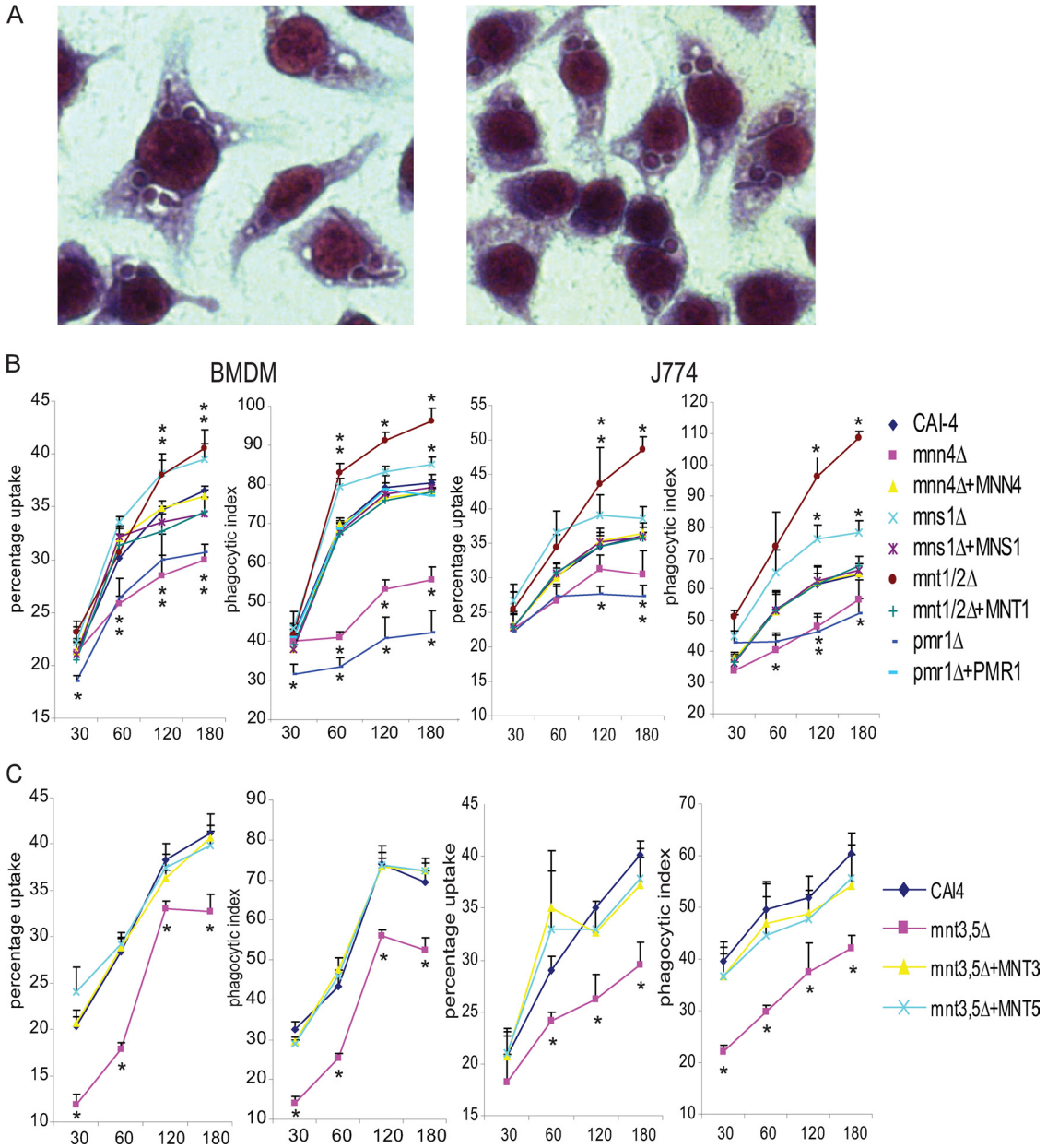

FIG. 1. Phagocytosis of *C. albicans* glycosylation mutant strains. (A) Light microscopy pictures of BMDM uptake of *C. albicans*. These pictures illustrate that not all macrophages ingest *C. albicans*, but those macrophages that ingest tend to take up more than one fungal cell. (B) Percentage uptake and phagocytic index for BMDM and J774 macrophages ingesting *C. albicans* glycosylation mutants (*mnn4*Δ, *mns*1Δ, *mnt1*Δ *mnt2*Δ, and *pmr1*Δ) and their respective reintegrant controls and the wild-type *C. albicans* strain (CAI4). The macrophage/*C. albicans* ratio was 1:1. Values are means ± standard deviations (SD); $n = 4$. *, $P < 0.05$. (C) Percentage uptake and phagocytic index for BMDM and J774 macrophages ingesting the *mnt3*Δ *mnt5*Δ mutant and the respective reintegrant controls (*mnt3*Δ *mnt5*Δ::*MNT3* and *mnt3*Δ *mnt5*Δ::*MNT5*) and wild-type *C. albicans* strain (CAI4). The macrophage/*C. albicans* ratio was 1:1. Values are means ± SD ($n = 4$). *, $P < 0.05$.

labeled overnight with 1 μl/ml of the green fluorescent probe FUN1 for 10 min then washed 3 times in 1% phosphate-buffered saline (PBS) to remove unbound FUN1 as per the manufacturer's instructions (Invitrogen). *C. albicans* strain serotype A strain CAI4, hitherto referred to as the wild type, was used as a control and to construct mutants using targeted gene disruption (26). The mutants are listed in Table 1. *C. albicans* strains containing a single reintegrated copy of the corresponding deleted genes were cultured and grown in the same way. These acted as reintegrant controls, to rescue part of the control phenotype. Most of the *C. albicans* strains used were created locally and have been described previously (1, 15, 16, 24, 25). The hypha-specific G_1 cyclin-null *hgc1*Δ mutant, the cyclic AMP (cAMP) pathway component regulator of hyphal morphogenesis *efg1*Δ mutant, the mitogen-activated protein (MAP) kinase transcription factor *cph1*Δ mutant, the B-type mitotic cyclin *clb2*Δ mutant, the *als3*Δ and *hwp1*Δ adhesin mutants, and the nonessential hypha-regulated cell wall protein *ece1*Δ mutant were all kindly provided by the authors of the references cited in Table 1.

Yeast cell phagocytosis assay. Primary BMDM and the macrophage cell lines J774.1 were plated at a density of 5×10^5 in 12-well plates (Nagle Nunc) for 24 h. Wild-type and mutant *C. albicans* strains from the overnight cultures were counted and added to BMDM and J774.1 macrophages at 1:1 and 3:1 *C. albicans*/macrophage ratios.

After 30, 60, 120, and 180 min of coincubation of J774.1 or BMDM and *C. albicans* strains, wells were washed twice with 1% (wt/vol) sterile phosphate-

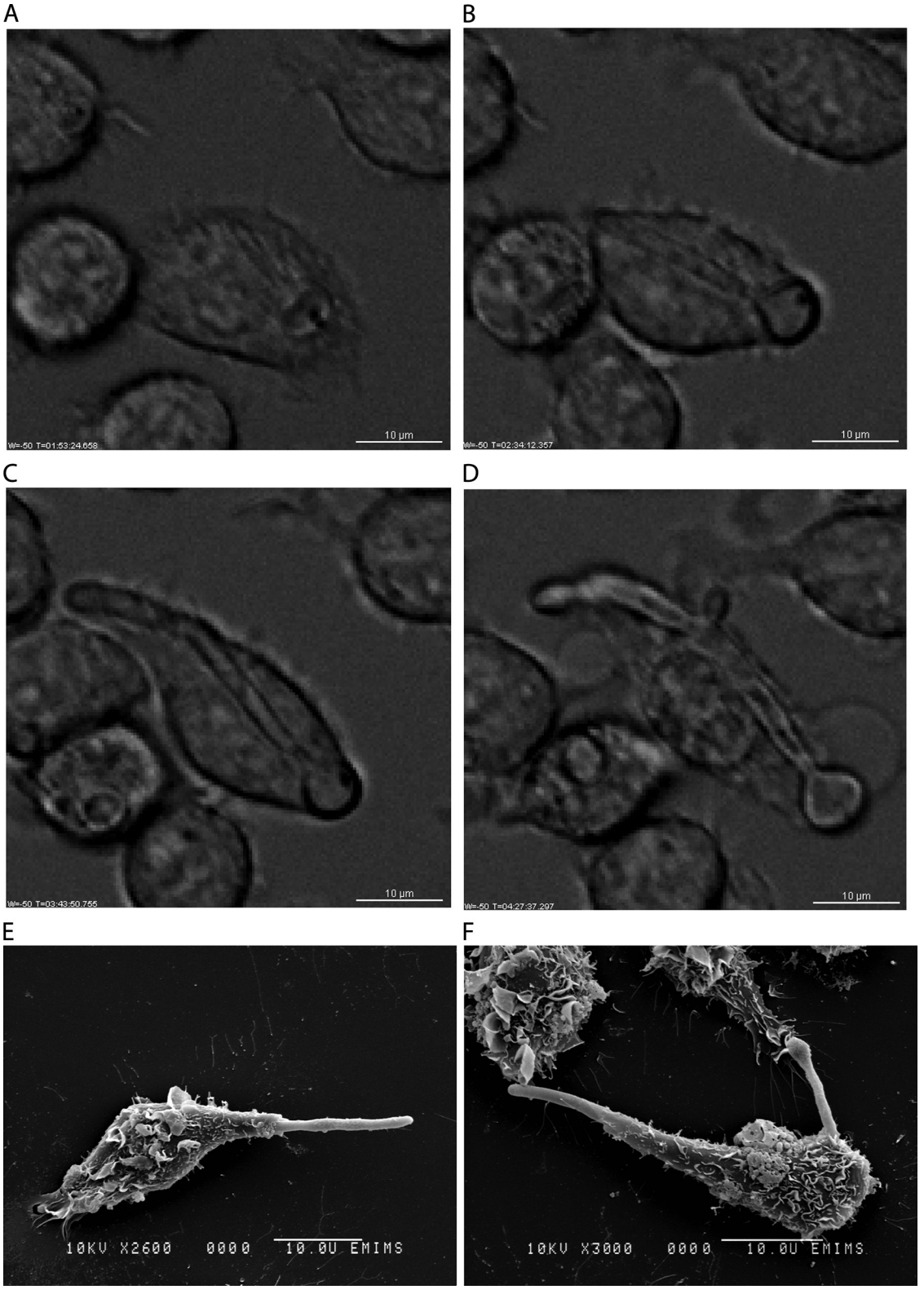

FIG. 2. Visualization of *C. albicans* hypha-mediated macrophage killing. Shown is video microscopy of J774 macrophages after ingestion of wild-type *C. albicans*. (A) Ingested *C. albicans* cells beginning to form hyphae within the macrophage. (B) Hyphal elongation (41 min post-image A). (C) Hypha stretching macrophage plasma membrane (1 h 50 min post-image A). (D) Hyphae piercing macrophage membrane and macrophage lysis (2 h 34 min post-image A). Shown are scanning electron micrographs of J774 macrophages and wild-type *C. albicans*. (E) This image shows a single *C. albicans* hypha protruding from a macrophage. (F) Hyphae protruding from macrophage triggering membrane ruffling and chemotaxis of neighboring macrophages.

TABLE 2. Percentage uptake of *C. albicans* cell wall and hypha-deficient mutants by macrophages

Ratio	% Uptake of *C. albicans* mutant by macrophages[a]					
	CAI4	*ece1*Δ	*als3*Δ	*als3*Δ::*ALS3*	*hwp1*Δ	*hwp1*Δ::*HWP1*
3:1	55.0 ± 4.8	60.7 ± 6	53.3 ± 1.2	52.3 ± 8.6	51 ± 10.8	59 ± 14.2
	NGY152CAI4	*clb2*Δ	*hgc1*Δ	*efg1*Δ	*cph1*Δ	*efg1*Δ *cph1*Δ
1:1	29.3 ± 3.5	31.3 ± 8.1	21.7 ± 3.8	35.3 ± 5.1	26.3 ± 0.6	32.7 ± 9.3
3:1	43.3 ± 4.5	50.3 ± 3.5	43.7 ± 2.5	42.3 ± 4.2	46.3 ± 1.5	44 ± 5.3

[a] The values shown are means ± standard deviations from three separate triplicate experiments. Data are shown for the 3-h time point at 1:1 and 3:1 *C. albicans*/J774 macrophage ratios.

buffered saline (PBS) to remove excess unbound cells. *C. albicans* uptake by macrophages was assessed by light microscopy (Nikon Eclipse TE2000-U microscope with a ×40 objective) in triplicate after fixation and staining using a modified Wright Giemsa stain (Laboratory Services, Paisley, United Kingdom) as previously described (10, 21). Results are expressed as percentage uptake (the percentage of macrophages that have taken up at least one fungal cell) and phagocytic index (the number of fungal cells taken up per 100 macrophages). Data were obtained in triplicate from at least 3 separate experiments by analyzing at least 200 macrophages per well.

Macrophage killing assay. The macrophage killing assay was conducted under the same conditions described above for the phagocytosis assay. After removal of excess unbound *C. albicans* by rigorous washing with 1% (vol/vol) PBS, killing of macrophages was assessed by trypan blue exclusion. A 150-μl sample of trypan blue (Sigma) and 150 μl of 1% PBS were added to cells for 2 min and removed by lightly washing twice with 1% PBS; cells were then fixed with 3% paraformaldehyde (BDH). Cells were then counted under an inverted light microscope (Nikon Eclipse TE2000-U microscope with a ×40 objective) to ascertain the percentage of macrophages killed. Data were obtained in triplicate from at least 3 separate experiments by analyzing at least 200 macrophages per well.

Scanning electron and live video microscopy. Circular glass slides (13 mm) were incubated overnight with 5×10^5 J774 macrophages in 24-well plates before addition of *C. albicans* control strain CAI4+CIP10, as described above. These were left to coincubate for 3 h and were then washed in 1% PBS and fixed with 150 μl of 2.5% grade 2 gluteraldehyde (Sigma). The glass slides were then observed using scanning electron microscopy (JEOL 35CF scanning electron microscope) at a ×2,600 magnification, with an accelerating voltage of 10 kV. Live video microscopy was conducted using a Deltavision Core microscope under the same conditions described for the phagocytosis assay. Images were taken every minute over a 6-h period at a ×40 magnification and analyzed using softWoRx Explorer image analysis software (Applied Precision, Issaquah, WA).

Statistical analysis. Each experiment was carried out at least 3 times in triplicate, and mean values and standard deviations were calculated. One-way analysis of variance and Mann-Whitney rank sum tests (Sigmaplot 11.0) were used to determine statistical significance.

RESULTS

Phagocytosis of glycosylation-deficient *C. albicans* strains. Mannoproteins on the *C. albicans* outer cell wall are thought to be the first point of contact between pathogen and cells of the innate immune system. This raises the question as to whether alterations in *C. albicans* cell wall glycosylation affect the rate of recognition and phagocytosis by macrophages.

We challenged primary macrophages and macrophage cell lines with glycosylation-defective strains of *C. albicans*. The strains used in this study are shown in Table 1. Briefly, the *pmr1*Δ strain lacks phosphomannan and has truncated O-linked and N-linked glycans; the *mnt1*Δ *mnt2*Δ strain is selectively deficient in O glycosylation (25) and has only a single O-linked mannose sugar; the *mns1*Δ strain has an N-glycosylation defect due to curtailed α1,2-mannosidase activity in the endoplasmic reticulum (24); the *mnn4*Δ strain has a complete loss of phosphomannan (16); and the *mnt3*Δ *mnt5*Δ strain has a partial phosphomannan alteration, as well as some minor alternations in *N*-mannan (15).

Uptake of *C. albicans* by macrophages was assessed using our standard phagocytosis assay, which we have used extensively in the past to quantitatively study ingestion of apoptotic, necrotic, and opsonized cells, as well as beads and other pathogens (9, 10, 21). Primary BMDM and the macrophage cell line J774.1 efficiently took up *C. albicans*, with most of the uptake occurring within the first 60 min of the interaction assay. However, only a proportion of macrophages in any given population ingested *C. albicans*, but those cells tended to ingest more than one fungal cell (Fig. 1A). This is similar to previous observations using other target cells (10). There were significant differences in the rate of uptake between control and mutant strains, which were most striking at the 3-h time point. The *mnt1*Δ *mnt2*Δ and *mns1*Δ strains were taken up at a significantly higher rate than the wild-type and reintegrant controls, whereas the phosphomannan-deficient *pmr1*Δ and *mnn4*Δ mutant strains showed significantly reduced rates of uptake (Fig. 1B). The importance of phosphomannan cell wall content for *C. albicans* recognition by macrophages was confirmed in experiments using the *mnt3*Δ *mnt5*Δ mutant strain (Fig. 1C), which has an ~50% reduction in phosphomannan.

Importantly, these observations were independent from the type of phagocyte (BMDM or cell line) and whether the assay was conducted under serum or serum-free conditions (data not shown). This confirms that the *C. albicans* glycosylation status is an important determinant for recognition and ingestion by macrophages and suggests that defects in phosphomannan affect the rate of uptake of yeast cells, whereas O-linked and N-linked mannosylation defects enhance pathogen recognition.

Macrophage killing by *C. albicans*. Morphogenic switching from yeast to hyphal forms has previously been thought to play a major role in *C. albicans*' ability to kill macrophages. It was therefore important to visualize the macrophage killing process in more detail. First, we conducted live video microscopy experiments which strongly suggest that killing of macrophages in the interaction assay is a consequence of hyphal formation, elongation, and ultimately stretching and piercing of the macrophage cell membrane (Fig. 2A to D). This is most strikingly illustrated by scanning electron microscopy pictures of killed macrophages, which show the piercing of the macrophage cell membrane by *C. albicans* hyphae (Fig. 2E and F).

Killing of macrophages by *C. albicans* glycosylation-deficient strains. To examine killing of macrophages by *C. albicans*, we again used glycosylation-deficient strains and further mutants that were deficient in hypha-specific cell wall proteins and others with deletions of genes coding for various regulatory proteins and transcription factors essential for the yeast-to-

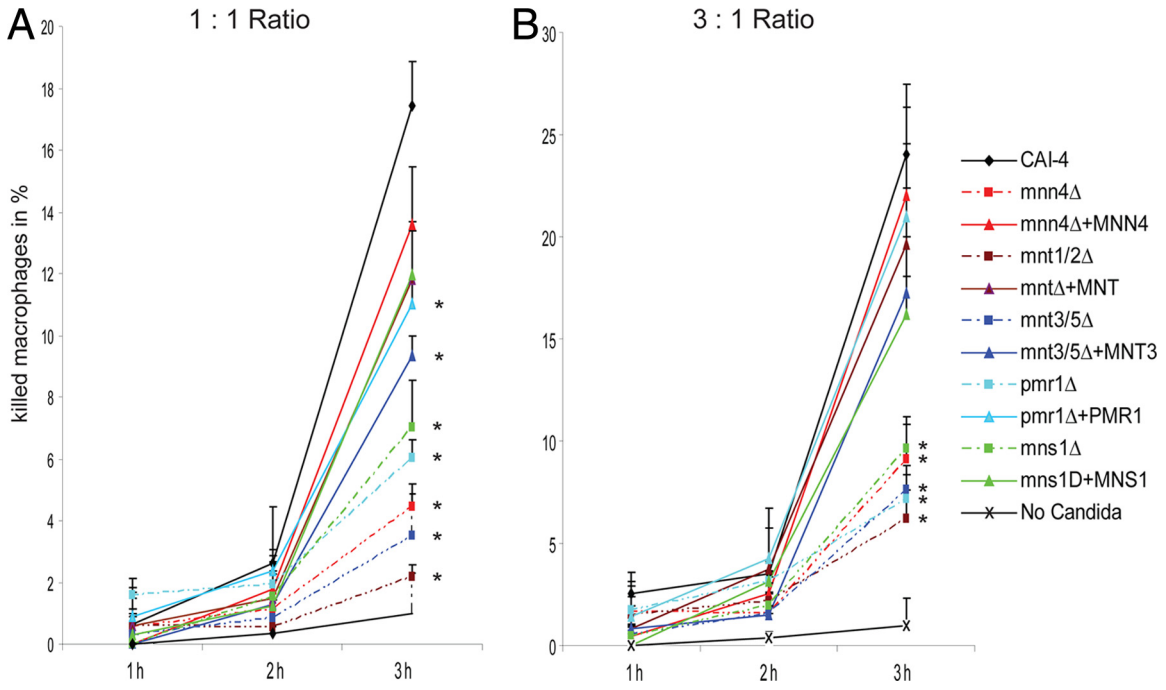

FIG. 3. Killing of macrophages by *C. albicans* glycosylation mutant strains. (A) Percentage of J774 macrophages killed by glycosylation mutants (*mnn4*Δ, *mns1*Δ, *mnt1*Δ *mnt*2Δ, and *pmr1*Δ), their respective reintegrant controls, and the wild-type *C. albicans* strain (CAI4). The *C. albicans*/macrophage ratio is 1:1 ($n = 4$). (B) Data for a *C. albicans*/macrophage ratio of 3:1 ($n = 4$) Values in panels A and B are means ± SD. *, $P < 0.05$.

hypha transition (Table 1). Morphogenesis-defective strains included the *clb2*Δ strain, a mitotic cyclin mutant that forms elongated buds, rather than true hyphae; the *hgc1*Δ strain, a G_1 cyclin mutant that is unable to form true hyphae; single and double *efg1*Δ mutant strains;, and *cph1*Δ mutants that lack specific transcription factors that regulate the yeast-hypha morphogenesis pathways (3, 19, 44).

Control phagocytosis experiments were conducted using yeast cells that had normal glycosylation but lacked specific hyphal cell wall proteins. As expected, mutants such as the *hwp1*Δ and *als3*Δ strains and the *ece1*Δ strain, which lack proteins only in the hypha cell wall, were unaffected in the rate of yeast cell uptake compared to wild-type controls (Table 2). Yeast cells of hypha-deficient mutants such as the *clb2*Δ, *hgc1*Δ, *efg1*Δ, and *cph1*Δ strains were also unaffected in the rate of uptake by macrophages compared to wild-type controls at 1 h and 3 h (Table 2).

Figure 3 shows the percentage of macrophages killed following incubation with control and glycosylation-deficient strains. There was little killing of macrophages during the first 2 h of the macrophage interaction assay but significant killing at 3 h. The *C. albicans*/macrophage ratio was a critical parameter for macrophage killing, with a higher rate of killing when using a 3:1 rather than a 1:1 *C. albicans*/macrophage ratio. All glycosylation-deficient strains showed a reduced rate of macrophage killing compared to the wild-type controls or their respective reintegrant strains, and this was observed at both 1:1 and 3:1 ratios (Fig. 3), but the biggest differentials between such strains occurred in assays using a 1:1 ratio of cells.

To further elucidate which glycosylation-deficient strains were most effective in killing macrophages, it was important to take into account the various rates of uptake of the individual strains. This analysis showed that the *mnt1*Δ *mnt2*Δ strain exhibited a much lower rate of macrophage killing than all other mutant strains tested (Table 3).

The *hwp1*Δ, *als3*Δ, and *ece1*Δ mutants that lacked specific cell wall proteins but were normal for yeast-hypha morphogenesis were unaffected in terms of macrophage killing compared to the wild-type or reintegrant controls (Table 4).

Next, we examined killing of macrophages by hypha-deficient strains. Strikingly, the *hgc1*Δ, *cph1*Δ, *efg1*Δ, *efg1*Δ *cph1*Δ, and *clb2*Δ mutant strains were all defective in macrophage killing. The *cph1*Δ mutant strain, which exhibits only a partial inhibition of hyphal formation, was associated with a 50%

TABLE 3. Macrophage killing rate by *C. albicans* glycosylation mutants

Ratio	% of macrophages killed by strain type[a]:									
	CAI4	*mnn4*Δ	*MNN4*	*mnt1*Δ *mnt2*Δ	*MNT1*	*pmr1*Δ	*mns1*Δ	*MNS1*	*mnt3*Δ[ι] *mnt5*Δ	*MNT3*
1:1	48.4	14.8	37.8	4.4	32.8	22.4	18.0	33.2	12.0	25.3

[a] The values shown represent the percentage of macrophages that were killed at the 3-h time point following ingestion of wild-type or mutant *C. albicans* at a 1:1 *C. albicans*/macrophage ratio. Each value represents the percentage of macrophages killed divided by the percentage uptake of *C. albicans* multiplied by 100.

TABLE 4. Percentage killing of J774 macrophages by *C. albicans* cell wall protein mutants

Ratio	% of macrophages killed by strain type[a]:						
	NGY152	*ece1*Δ	*als3*Δ	*als3*Δ::*ALS3*	*hwp1*Δ	*hwp1*Δ::*HWP1*	Macrophages only
1:1	9.77 ± 3.06	9.44 ± 4.29	9.29 ± 2.52	9.15 ± 3.75	9.35 ± 1.57	8.87 ± 0.78	0.96
3:1	20.01 ± 3.37	18.01 ± 7.05	19.21 ± 4.36	18.76 ± 2.35	19.09 ± 0.73	18.41 ± 1.22	0.96

[a] The values shown represent the percentage of macrophages killed by *C. albicans* ± standard deviation. Shown are the results from 3 separate triplicate experiments at the 3-h time point for 1:1 and 3:1 *C. albicans*/macrophage ratios.

reduction in macrophage killing, while the other mutants with more severe morphogenesis deficiencies were even more defective in macrophage killing (Table 5). This suggests that hyphal formation is essential for the ability of *C. albicans* to kill macrophages. This is further supported by fluorescence microscopy experiments in which macrophages were exposed to the fluorescent stain FUN1. Staining macrophages with FUN1 resulted in marked inhibition of hyphal formation and a significant reduction in macrophage killing (CAI4 versus CAI4 plus FUN1, 17.4% ± 1.46% versus 7.3% ± 1.71%, respectively; $n = 4$; $P < 0.05$) without alteration in macrophage uptake (percentage uptake for CAI4 versus CAI4 plus FUN1, 35.1% ± 2.87% versus 36.5% ± 4.01%, respectively; $n = 3$).

Finally, we confirmed previous observations that hypha formation was not affected in the *C. albicans* glycosylation mutants (16, 25) (Table 6). Furthermore, most glycosylation mutants were also unaffected in hyphal formation after ingestion by macrophages. Only the *mns1*Δ null mutant showed a slight delay in hypha formation *in vitro* and within macrophages (Table 6). Thus, the reduced killing of macrophages by glycosylation mutants was not due to an impaired ability to form hyphae.

DISCUSSION

C. albicans is the major life-threatening human fungal pathogen. *C. albicans* pathogenicity depends in part on its ability to escape immune surveillance and to kill immunocompetent host cells. Recent studies have shown that components of the fungal cell wall such as β-glucan (33) and mannan (6) are immunostimulatory and are involved in the recognition and uptake of fungal cells by the immune system (27, 37).

In this study, we analyzed the contribution of distinct *C. albicans* cell wall components and yeast-hypha morphogenesis to phagocytosis by and escape from macrophages. We show that *C. albicans* phagocytosis by macrophages is dependent on the glycosylation status of the cell wall, but not cell wall proteins or morphogenic switching from yeast to hyphal forms. Morphogenic switching, however, is required for macrophage killing by *C. albicans*, and killing of macrophages is abrogated in mutants that are unable to form hyphae. Interestingly, glycosylation mutants exhibit normal hypha formation extracellularly in culture and after uptake within macrophage phagosomes but have diminished ability to kill and escape from phagocytes.

Recognition of unopsonized yeast cells of *C. albicans* by monocytes and macrophages is mediated by at least four recognition systems that sense specific components of the *C. albicans* cell wall (N-linked mannans by MR, O-linked mannans by TLR4, β-glucans by dectin-1, and β-mannosides by galectin-3/TLR2 complexes). These have been implicated by ourselves and others in experiments combining *C. albicans* cell wall mutants with tools to specifically block macrophage surface receptors, such as knockout BMDM, receptor-blocking antibodies, and ligands (28). These experiments have identified profound differences in the cytokine responses following uptake, depending on which recognition pathway was engaged. Here we show that the glycosylation status of the *C. albicans* cell wall profoundly affected the rate of macrophage phagocytosis. Distinct patterns emerged in that phosphomannan-deficient strains (*mnn4*Δ, *pmr1*Δ, and *mnt3*Δ *mnt5*Δ) were taken up at a lower rate than the wild-type or reintegrant controls and that O-linked and N-linked mannan-deficient strains are taken up at higher rates (*mns1*Δ and *mnt1*Δ *mnt2*Δ). Interestingly, the phosphomannan-deficient *pmr1*Δ strain shows reduced uptake, despite its deficiencies in O-linked and N-linked mannan. The *O*-mannan structure of the *pmr1*Δ mutant differs from that of the *mnt1*Δ *mnt2*Δ strain in having a less marked truncation of the glucan chain (1). This difference suggests the importance of terminal α-1,2 mannose in determining recognition and uptake of *C. albicans* yeast cells by macrophages. The cell wall phosphomannan content plays an important role in the surface charge of the *C. albicans* cell wall, and the findings described here mirror recent observations made in apoptotic and viable cells identifying surface charge as an important determinant for chemotaxis toward phagocytes (39, 43) and ultimately phagocytosis (10). This is in contrast with previous reports that failed to demonstrate a role for phosphomannan in phagocytosis assays over a shorter time frame using

TABLE 5. Percentage killing of macrophages by *C. albicans* hypha-deficient mutants

Ratio	% of macrophages killed by strain type[a]:						
	CAI4	*clb2*Δ	*hgc1*Δ	*efg1*Δ	*cph1*Δ	*efg1*Δ::*cph1*Δ	Macrophages only
1:1	10.2 ± 0.68	1.9 ± 0.78*	1.6 ± 1.44*	2.37 ± 1.33*	5.89 ± 1.02*	2.15 ± 1.97*	2.81 ± 2.83*
3:1	20.6 ± 2.95	1.83 ± 0.78*	2.5 ± 0.95*	2.46 ± 1.84*	11.62 ± 2.19*	2.19 ± 1.06*	2.81 ± 2.83*

[a] The values shown represent percentage of macrophages killed by *C. albicans* ± standard deviation. Shown are the results from 3 separate triplicate experiments at the 3-h time point for 1:1 and 3:1 *C. albicans*/macrophage ratios. *, significantly different ($P < 0.05$) compared to the wild-type control.

TABLE 6. Hyphal formation rate of *C. albicans* glycosylation mutants

Time	% of hyphal formation in strain type[a]:						
	CAI4	*mnn4*Δ	*mnn4*Δ::*MNN4*	*mnt1*Δ *mnt2*Δ	*mnt1*Δ *mnt2*Δ::*MNT1*	*pmr1*Δ	*mns1*Δ
Extracellular							
30 min	74	83.5	69.83	91.67	85.33	60.12	67
1 h	95.99	86.83	96	94.94	95.33	85.17	83.2
2 h	100	100	100	100	100	97.34	100
3 h	100	100	100	100	100	100	100
Intracellular							
30 min	44.8	65.83	48.17	50.9	38.33	32	19.37
1 h	83.22	87.17	81.17	84.33	83	73.75	67.5
2 h	97.5	95.49	96.38	93.55	95.33	95.34	76.77
3 h	100	100	100	100	100	100	100

[a] The values shown represent the percentage of *C. albicans* over time that form hyphae extracellularly and after ingestion by macrophages.

a much higher *C. albicans*/macrophage ratio (16). The conditions used in the previous study may have overshadowed the differences between the wild-type and *mnn4*Δ strains revealed here.

The absence of O-linked or N-linked mannans significantly increased the phagocytosis of *C. albicans*. Alcian blue assays confirm (data not shown) that this is not a consequence of a compensatory increase in phosphomannan. A more likely interpretation is that these cell wall components play a direct role in virulence or a negative role in the recognition of this fungus by macrophages and other cells of the immune system (11, 14, 27, 41). Although cell wall mannans can mask recognition of underlying β-glucans in the cell wall, they also participate in immune recognition (27). Here we demonstrate that they are also required for phagocytosis and affect the rate of killing of macrophages once taken up into macrophage phagosomes.

C. albicans is a dimorphic fungus, and its ability to switch between yeast and hyphal forms is thought to contribute to pathogenesis (13). *C. albicans* mutants that are unable to form filaments are less virulent (17, 19), although conversely, mutants that are unable to grow as yeast are also less virulent (34), suggesting that both forms are needed for pathogenicity. There is evidence suggesting that yeast and hyphal forms engage different macrophage receptors during engulfment (11), and this is associated with alteration in the ensuing macrophage cytokine and chemokine profile (36). However, little is known about how impaired morphogenic switching affects recognition and uptake by macrophages and the ability of *C. albicans* to evade immune destruction and escape from macrophages after uptake (32).

The data shown here employing hypha-deficient mutant strains confirm the importance of morphogenic switching for *C. albicans* to kill and escape from macrophages. The live cell video and scanning electron microscopy images shown here support the notion that this is accomplished by physical perforation of the macrophage cell membrane. Importantly, our data show that even though the absence of hyphal formation abrogates macrophage killing, intact hyphal formation does not always result in efficient macrophage killing.

The data presented here shed light on why glycosylation-deficient mutants show profound defects in macrophage killing despite normal intraphagosomal hyphal formation. It appears likely that the engagement of distinct PRRs by specific glycosylation mutants during uptake and recognition, as described by Netea et al. (28, 29), alters the maturation of macrophage phagosomes. This in turn may expose the glycosylation mutants to a potentially more hostile environment and favor digestion of *C. albicans* rather than escape and macrophage killing. This is an area under active investigation in our laboratory, and differences in maturation of phagosome containing wild-type and mutant *C. albicans* strains would be directly relevant to other infectious microorganisms.

The experiments described here show that specific fungal properties are associated with defined effects on the macrophages' ability to ingest and process *C. albicans*. The work contributes to a better understanding of the innate immune response to infection and is a prerequisite for future studies linking pathogen recognition and phagosome maturation and its consequences for survival of the pathogen and host.

ACKNOWLEDGMENTS

L.P.E. thanks the Wellcome Trust for funding (073566). L.P.E. is a Scottish Senior Clinical Fellow and thanks the CSO for support. N.A.R.G. thanks the Wellcome Trust for funding (080088).

REFERENCES

1. **Bates, S., D. M. MacCallum, G. Bertram, C. A. Munro, H. B. Hughes, E. T. Buurman, A. J. Brown, F. C. Odds, and N. A. Gow.** 2005. Candida albicans Pmr1p, a secretory pathway P-type Ca2+/Mn2+-ATPase, is required for glycosylation and virulence. J. Biol. Chem. **280:**23408–23415.
2. **Bates, S., H. B. Hughes, C. A. Munro, W. P. Thomas, D. M. MacCallum, G. Bertram, A. Atrih, M. A. Ferguson, A. J. Brown, F. C. Odds, and N. A. Gow.** 2006. Outer chain *N*-glycans are required for cell wall integrity and virulence of *Candida albicans*. J. Biol. Chem. **281:**90–98.
3. **Bensen, E. S., A. Clemente-Blanco, K. R. Finley, J. Correa-Bordes, and J. Berman.** 2005. The mitotic cyclins Clb2p and Clb4p affect morphogenesis in *Candida albicans*. Mol. Biol. Cell **16:**3387–3400.
4. **Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd.** 1993. Cloning and characterization of ECE1, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. Infect. Immun. **61:**3648–3655.
5. **Cutler, J. E.** 2001. *N*-Glycosylation of yeast, with emphasis on *Candida albicans*. Med. Mycol. **39:**75–86.
6. **Dalle, F., T. Jouault, P. A. Trinel, J. Esnault, J. M. Mallet, P. d'Athis, D. Poulain, and A. Bonnin.** 2003. Beta-1,2- and alpha-1,2-linked oligomannosides mediate adherence of Candida albicans blastospores to human enterocytes in vitro. Infect. Immun. **71:**7061–7068.
7. **Dennehy, K. M., G. Ferwerda, I. Faro-Trindade, E. Pyż, J. A. Willment, P. R. Taylor, A. Kerrigan, S. V. Tsoni, S. Gordon, F. Meyer-Wentrup, G. J. Adema, B.-J. Kullberg, E. Schweighoffer, V. Tybulewicz, H.-M. Mora-Montes, N. A. R. Gow, D. L. Williams, M. G. Netea, and G. D. Brown.** 2008. Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur. J. Immunol. **38:**500–506.

8. **Ernst, J. F., and S. K. Prill.** 2001. *O*-Glycosylation. Med. Mycol. **39:**67–74.
9. **Erwig, L. P., D. C. Kluth, G. M. Walsh, and A. J. Rees.** 1998. Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. J. Immunol. **161:**1983–1988.
10. **Erwig, L. P., K. A. McPhilips, M. W. Wynes, A. Ivetic, A. J. Ridley, and P. M. Henson.** 2006. Differential regulation of phagosome maturation in macrophages and dendritic cells mediated by Rho GTPases and ERM proteins. Proc. Natl. Acad. Sci. U. S. A. **103:**12825–12830.
11. **Gantner, B. N., R. M. Simmons, and D. M. Underhill.** 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J. **24:**1277–1286.
12. **Goldman, D. L., S. C. Lee, and A. Casadevall.** 1995. Tissue localization of *Cryptococcus neoformans* glucuronoxylomannan in the presence and absence of specific antibody. Infect. Immun. **63:**3448–3453.
13. **Gow, N. A., A. J. Brown, and F. C. Odds.** 2002. Fungal morphogenesis and host invasion. Curr. Opin. Microbiol. **5:**366–371.
14. **Gow, N. A. R., M. G. Netea, C. A. Munro, G. Ferwerda, S. Bates, H. M. Mora-Montes, L. Walker, T. Jansen, L. Jacobs, V. Tsoni, G. D. Brown, F. C. Odds, J. W. M. Van der Meer, A. J. P. Brown, and B.-J. Kullberg.** 2007. Recognition of *Candida albicans* β-glucan by dectin-1 induces cytokines and has non-redundant effects on the activation of innate immunity. J. Infect. Dis. **196:**1565–1571.
15. **Harris, M., H. M. Mora-Montes, N. A. Gow, and P. J. Coote.** 2009. Loss of mannosylphosphate from *Candida albicans* cell wall proteins results in enhanced resistance to the inhibitory effect of a cationic antimicrobial peptide via reduced peptide binding to the cell surface. Microbiology **155:**1058–1070.
16. **Hobson, R. P., C. A. Munro, S. Bates, D. M. MacCallum, J. E. Cutler, S. Heinsbroek, G. D. Brown, F. C. Odds, and N. A. R. Gow.** 2004. Loss of cell wall phosphomannan in *Candida albicans* does not influence macrophage phagocytosis and function. J. Biol. Chem. **279:**39628–39635.
17. **Kadosh, D., and A. D. Johnson.** 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. Mol. Cell. Biol. **16:**2903–2912.
18. **Lee, S. J., S. Evers, D. Roeder, A. F. Parlow, J. Risteli, L. Risteli, Y. C. Lee, T. Feizi, H. Langen, and M. C. Nussenzweig.** 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. Science **295:**1898–1901.
19. **Lo, H. J., J. R. Köhler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
20. **Loeb, J. D., M. Sepulveda-Becerra, I. Hazan, and H. Liu.** 1999. A G1 cyclin is necessary for maintenance of filamentous growth in *Candida albicans*. Mol. Cell. Biol. **19:**4019–4027.
21. **McPhillips, K. A., and L. P. Erwig.** 2009. Assessment of apoptotic cell phagocytosis by macrophages. Methods Mol. Biol. **559:**247–256.
22. **Means, T. K., E. Mylonakis, E. Tampakakis, R. A. Colvin, E. Seung, L. Puckett, M. F. Tai, C. R. Stewart, R. Pukkila-Worley, S. E. Hickman, K. J. Moore, S. B. Calderwood, N. Hacohen, A. D. Luster, and J. El Khoury.** 2009. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. J. Exp. Med. **206:**637–653.
23. **Miyazato, A., K. Nakamura, Y. Abe, H.-M. Mora-Montes, K. Inden, X. Gang, K. Ishii, K. Takeda, S. Akira, S. Saijo, Y. Iwakura, Y. Adachi, N. Ohno, N. A. R. Gow, M. Kaku, and K. Kawakami.** 2009. Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from Candida albicans. Infect. Immun. **74:**3056–3064.
24. **Mora-Montes, H. M., S. Bates, M. G. Netea, D. F. Díaz-Jiménez, E. López-Romero, S. Zinker, P. Ponce-Noyola, B. J. Kullberg, A. J. P. Brown, F. C. Odds, A. F. Carreón, and N. A. R. Gow.** 2007. The endoplasmic reticulum α-glycosidases of *Candida albicans* are required for *N*-glycosylation, cell wall integrity, and normal host-fungus interaction. Eukaryot. Cell **6:**2184–2193.
25. **Munro, C. A., S. Bates, E. T. Buurman, H. B. Hughes, A. Atrih, M. A. J. Ferguson, D. M. MacCallum, G. Bertram, J. M. Bain, A. Brand, C. Westwater, L. M. Thomson, A. J. P. Brown, F. C. Odds, and N. A. R. Gow.** 2005. Mnt1p and Mnt2p of *Candida albicans* are functionally redundant α-1,2-mannosyltransferases that participate in *O*-linked mannosylation and are required for adhesion and virulence. J. Biol. Chem. **280:**1051–1060.
26. **Murad, A. M., P. R. Lee, I. D. Broadbent, C. J. Barelle, and A. J. Brown.** 2000. CIp10, an efficient and convenient integrating vector for Candida albicans. Yeast **16:**325–327.
27. **Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. R. Gow.** 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat. Rev. Microbiol. **6:**67–78.
28. **Netea, M. G., N. A. R. Gow, C. A. Munro, S. Bates, C. Collins, G. Ferwerda, R. P. Hobson, G. Bertram, H. B. Hughes, T. Jansen, L. Jacobs, E. T. Buurman, K. Gijzen, D. L. Williams, R. Torensma, J. W. M. Van der Meer, A. McKinnon, F. C. Odds, A. J. P. Brown, and B. J. Kullberg.** 2006. Immune sensing of *Candida albicans*: cooperative recognition of mannans and glucan by lectin and Toll-like receptors. J. Clin. Invest. **6:**1642–1650.
29. **Netea, M. G., C. A. Van Der Graaf, A. G. Vonk, I. Verschueren, J. W. Van Der Meer, and B. J. Kullberg.** 2002. The role of toll-like receptor (TLR) 2 and TLR4 in the host defence against disseminated candidiasis. J. Infect. Dis. **185:**1483–1489.
30. **Nobile, C. J., J. E. Nett, D. R. Andes, and A. P. Mitchell.** 2006. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. Eukaryot. Cell **5:**1604–1610.
31. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biol. **5:**e64.
32. **Romani, L.** 2004. Immunity to fungal infections. Nat. Rev. Immunol. **4:**1–23.
33. **Rubin-Bejerano, I., C. Abeijon, P. Magnelli, P. Grisafi, and G. R. Fink.** 2007. Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. Cell Host Microbe **2:**55–67.
34. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. Eukaryot. Cell **2:**1053–1060.
35. **Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown.** 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat. Immunol. **8:**31–38.
36. **Torosantucci, A., P. Chiani, and A. Cassone.** 2000. Differential chemokine response of human monocytes to yeast and hyphal forms of *Candida albicans* and its relation to the beta-1,6 glucan of the fungal cell wall. J. Leukoc. Biol. **68:**923–932.
37. **van de Veerdonk, F. L., R. J. Marijnissen, B. J. Kullberg, H. J. Koenen, S. C. Cheng, I. Joosten, W. B. van den Berg, D. L. Williams, J. W. van der Meer, L. A. Joosten, and M. G. Netea.** 2009. The macrophage mannose receptor induces IL-17 in response to Candida albicans. Cell Host Microbe **5:**329–340.
38. **Vecchiarelli, A., M. Dottorini, D. Pietrella, C. Monari, C. Retini, T. Todisco, and F. Bistoni.** 1994. Role of human alveolar macrophages as antigen-presenting cells in *Cryptococcus neoformans* infection. Am. J. Respir. Cell Mol. Biol. **11:**130–137.
39. **Weihua, Z., R. Tsan, A. J. Schroit, and I. J. Fidler.** 2005. Apoptotic cells initiate endothelial cell sprouting via electrostatic signaling. Cancer Res. **65:**11529–11535.
40. **Wells, C. A., J. A. Salvage-Jones, X. Li, K. Hitchens, S. Butcher, R. Z. Murray, A. G. Beckhouse, Y. L. Lo, S. Manzanero, C. Cobbold, K. Schroder, B. Ma, S. Orr, L. Stewart, D. Lebus, P. Sobieszczuk, D. A. Hume, J. Stow, H. Blanchard, and R. B. Ashman.** 2008. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. J. Immunol. **180:**7404–7413.
41. **Wheeler, R. T., and G. R. Fink.** 2006. A drug-sensitive genetic network masks fungi from the immune system. PLoS Pathog. **2:**e35.
42. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. **39:**309–317.
43. **Zhao, M., B. Song, J. Pu, T. Wada, B. Reid, G. Tai, F. Wang, A. Guo, P. Walczysko, Y. Gu, T. Sasaki, A. Suzuki, J. V. Forrester, H. R. Bourne, P. N. Devreotes, C. D. McCaig, and J. M. Penninger.** 2006. Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. Nature **442:**457–460.
44. **Zheng, X., Y. Wang, and Y. Wang.** 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. EMBO J. **23:**1845–1856.

Editor: G. S. Deepe, Jr.